Effect of Sodium Nitrite on Growth of *Shigella flexneri*

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ABSTRACT

A partial factorial design study of the effect of $NaNO_2$ (0, 100, 200, 1000 ppm) in combination with NaCl (0.5, 2.5, 4.0%), pH (7.5, 6.5, 5.5), and temperature (37, 28, 19°C) on growth of *Shigella flexneri* is reported. Experiments were done aerobically in brain-heart infusion medium, using an inoculum of 1 x 10^3 CFU/ml. Growth curves were fitted from plate count data by the Gompertz equation; exponential growth rates, lag times, generation times, and maximum populations were derived for all variable combinations. In the absence of nitrite, the organism grew well under all test conditions at 37 and 28°C but did not grow at 19°C at pH 5.5 nor at pH 7.5 with 4% NaCl. Nitrite did not affect growth in media of pH 7.5 at 37 and 28°C. At pH 6.5 growth was inhibited by 1000 ppm $NaNO_2$. The organism failed to grow at 19°C at all nitrite levels in the presence of 2.5 or 4.0% NaCl. The inhibitory effect of nitrite was much greater in media of pH 5.5 and increased with increasing salt levels. More inhibition was apparent at 28 than at 37°C. While lack of growth was used as a paradigm of the effect of nitrite on *S. flexneri*, nitrite also increased the lag and generation times and decreased the exponential growth rate. Results indicated that $NaNO_2$ in combinations with low temperature, low pH, and high salt content can effectively inhibit the growth of *S. flexneri*.

Shigella has been recognized in recent years as one of the major causative agents of foodborne gastrointestinal disease (*5,12*). Although considerable attention has been given to investigations of the taxonomy, epidemiology, and virulence of *Shigella* species (*12*), few systematic studies have been carried out on the effects of various environmental and cultural conditions on the viability and growth of *Shigella*. Fehlhaber (*4*) reported on the temperature, pH, and sodium chloride concentration ranges that permitted the growth of 21 strains of *S. flexneri* and 21 strains of *S. sonnei*. The action of nitrite on *Shigella* has received limited attention. Fehlhaber (*4*) reported inhibition of 21 strains of *S. flexneri* by nitrite in media of low pH. Although only limited studies have been carried out on the survival of *Shigella* in foods (*11,14*), there are indications that these bacteria are capable of surviving for considerable lengths of time even in acidic foods (*11*). Since the infectious dose is reported (8) to be quite low (10-100 organisms), it is essential to determine the role that food ingredients, processing parameters, and environmental conditions or storage conditions may play in determining the fate of *Shigella* in food and its ability to cause infection.

Recently we reported (*15*) on the effect of sodium chloride (0.5-5.0%), pH (5.5-7.5), and temperature (10-37°C) on growth of *S. flexneri*. The purpose of this investigation was to expand the previous work to determine the effect of sodium nitrite under various conditions of sodium chloride concentration, pH, and temperature on *S. flexneri* cultured in a microbiological medium in order to provide needed basic information which would be useful in predicting the behavior of *Shigella* species in food.

EXPERIMENTAL

Microorganism

Shigella flexneri 5348 (obtained from Dr. David W. Niesel, University of Texas Medical Branch, Galveston, TX) was used throughout the study. To prepare the inoculum, the organism was cultured for 24 h in brain-heart infusion (Difco, Detroit, MI) at 37°C, and the culture was diluted with sterile 0.1% peptone water.

Experimental design

A partial factorial design was employed (3 x 3 x 3 x 4 x 3) to assess the effects of temperature (37, 28, 19°C), pH (5.5, 6.5, 7.5), sodium chloride (0.5, 2.5, 4.0%), and sodium nitrite (0, 100, 200, 1000 ppm). All variable combinations were replicated three times.

Culture technique

Brain-heart infusion broth (Difco), diluted to 90% of final volume, was supplemented with 0, 20 or 35 g sodium chloride/900 ml; pH was adjusted to 7.5, 6.5, or 5.5 using lN NaOH or lN HCl. The medium was then dispensed in 90-ml portions into 500-ml Erlenmeyer flasks. The flasks were capped with foam plugs and sterilized by autoclaving for 15 min at 15 psi at 121°C. Filter-sterilized sodium nitrite solutions (0, 1000, 2000, or 10000 ppm) were added in 10-ml volumes to the sterile media. The final media contained 0.5, 2.5, or 4.0% sodium chloride and 0, 100, 200, or 1000 ppm (micrograms/ml) sodium nitrite. All flasks were inoculated with 1 ml of a diluted 24-h culture of *S. flexneri* to an initial level of approximately 1 x 10^3 CFU/ml. All flasks were then incubated on a rotary shaker (150 rpm) at the desired temperature.

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TABLE 1. *Gompertz equation.*

$$L(t)=A + C \exp [- \exp (- B (t - M))]$$

L(t) = the log count of the number of bacteria at time t (in hours).
A = the asymptotic log count as t decreases indefinitely.
C = the asymptotic amount of growth (log number) that occurs as t increases indefinitely.
M = the time (in hours) at which the absolute growth rate is maximum.
B = the relative growth rate at M.

Associated Equations:

Exponential growth rate [($\log_{10}$ CFU/ml)/h] = BC/e
Lag phase duration (h) = M - (1/B)
Generation time (h) = ($\log_{10}$ 2e)/BC
Maximum population density ($\log_{10}$ CFU/ml) = A + C

At appropriate intervals, samples were withdrawn from each flask by means of a pipet and the microbial population determined by surface plating on tryptic soy agar (Difco) using a Spiral Plater (Spiral System Instruments, Inc.. Bethesda, MD). The plates (in duplicate) were incubated for 24 h at 37°C and counted.

Curve fitting and statistical analyses

Growth curves were generated from the experimental data using the Gompertz equation (Table l) in conjunction with ABACUS, a nonlinear regression program that employs a Gauss-Newton iteration procedure. This FORTRAN-based program was developed by W. C. Damert (U.S. Department of Agriculture, Eastern Regional Research Center, Philadelphia, PA), and copies are available upon request. The Gompertz parameter values (A, B, C, M) were subsequently used to calculate lag times (h), exponential growth rates [($\log_{10}$ CFU/ml)/h], generation times (h), and maximum population densities ($\log_{10}$CFU/ml) as described by Gibson et al. (*6*).

RESULTS

Bacterial growth curves were calculated from the experimental data for each variable combination using the Gompertz equation (*6*) (Table 1). The Gompertz equation parameters (A, B, C, M) obtained were subsequently used to calculate exponential growth rates, generation times, lag times, and maximum population densities (Table 2) for all the variable combinations tested.

In the absence of nitrite the maximum growth rate was obtained at 37°C and growth rates in cultures of pH 5.5 were lower than in cultures of pH 6.5 and 7.5. Increasing levels of sodium chloride progressively decreased growth rates and increased generation times and lag times. However, when growth occurred, the maximum population densities were high, even in the presence of up to 4.0% sodium chloride.

In the presence of nitrite, growth inhibition was highly dependent on the pH and the salt content of the medium and on the temperature of incubation.

37°C. *S. flexneri* grew well at pH 7.5 regardless of nitrite or salt levels. Growth was obtained in media of pH 6.5 containing 0.5 or 2.5% NaCl except in the presence of 1000 ppm $NaNO_2$. The degree of inhibition by nitrite was much greater at pH 5.5, particularly in combination with increased levels of sodium chloride. Concentrations of 1000, 200, and 100 ppm $NaNO_2$ were inhibitory in media containing 0.5, 2.5, and 4.0% NaCl, respectively. Increasing the nitrite level led to an increase in lag time, generation time and a decrease in exponential growth rate, particularly at lower pH and higher salt levels.

28°C. The growth pattern of *S.flexneri* cultures at 28°C was similar to that obtained at 37°C in media of pH 6.5 and 7.5. At pH 5.5 bacterial growth was inhibited by 200 and 1000 ppm $NaNO_2$ in media containing 0.5% NaCl

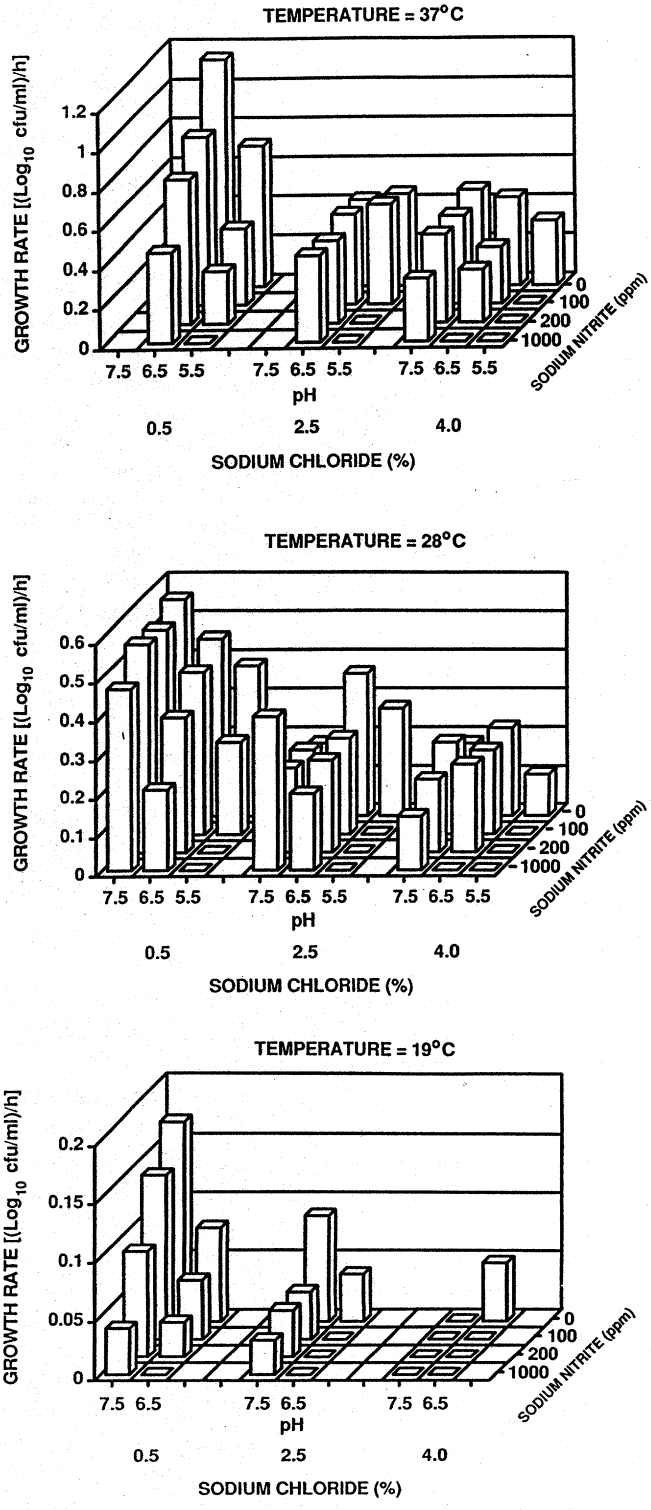

Figure 1. *Exponential growth rates of* S. flexneri *in BHI media under various conditions of pH (7.5, 6.5, and 5.5), NaCl content (0.5, 2.5, and 4.0%), and $NaNO_2$ content 0, 100, 200, and 1000 ppm) at 37°C (a), 28° C (b), and 19° C (c).*

TABLE 2.

Temp. (° C)	pH	NaCl (%)	$NaNO_2$ (ppm)	A	C	B	M	Growth rate (log_{10} CFU/ml)/h	Generation time h	Lag time h	Max. pop. density log_{10} CFU/ml
28	7.5	0.5	0	2.66	7.23	0.212	8.95	0.56	0.5	4.2	9.9
			100	2.66	7.26	0.198	9.25	0.53	0.6	4.2	9.9
			200	2.66	7.26	0.201	9.01	0.54	0.6	4.0	9.9
			1000	2.66	7.08	0.180	9.47	0.47	0.6	3.9	9.8
		2.5	0	2.65	6.46	0.081	17.52	0.19	1.6	5.1	9.1
			100	2.65	6.77	0.083	18.08	0.22	1.4	6.9	9.4
			200	2.65	5.05	0.127	12.14	0.28	1.1	4.3	8.7
			1000	2.65	6.03	0.180	9.36	0.40	0.8	3.8	8.7
		4.0	0	3.38	6.17	0.086	54.58	0.19	1.6	42.9	9.6
			100	2.66	6.34	0.104	48.75	0.24	1.2	39.1	9.0
			200	3.07	5.93	0.087	44.15	0.19	1.6	32.7	9.0
			1000	3.25	5.61	0.068	50.50	0.14	2.1	35.9	8.9
19	6.5	0.5	0	2.87	7.02	0.032	39.32	0.08	3.7	7.8	9.9
			100	2.93	7.10	0.018	80.05	0.05	6.6	22.9	10.0
			200	3.08	6.66	0.014	326.65	0.03	9.1	252.6	9.7
			1000	2.87	NG	0.000	-	-	-	-	-
		2.5	0	2.97	6.87	0.011	114.99	0.04	7.1	55.1	9.8
			100	2.97	NG	0.000	-	-	-	-	-
			200	2.97	NG	0.000	-	-	-	-	-
			1000	2.97	NG	0.000	-	-	-	-	-
		4.0	0	2.70	6.12	0.021	183.11	0.05	6.3	135.1	8.8
			100	2.61	NG	0.000	-	-	-	-	-
			200	2.61	NG	0.000	-	-	-	-	-
			1000	2.61	NG	0.000	-	-	-	-	-
19	7.5	0.5	0	2.87	7.52	0.063	77.29	0.17	1.7	6.2	10.4
			100	2.81	7.50	0.049	78.38	0.14	2.2	58.0	10.3
			200	2.90	7.50	0.031	85.13	0.09	3.5	53.3	10.4
			1000	3.11	6.74	0.014	143.07	0.04	8.5	73.1	9.9
		2.5	0	2.98	6.47	0.038	81.80	0.09	3.3	55.4	9.4
			100	3.41	6.13	0.017	189.46	0.04	7.8	130.6	9.5
			200	3.48	6.23	0.020	222.77	0.04	6.7	171.5	9.7
			1000	2.81	5.92	0.015	314.22	0.03	9.0	249.3	8.7
		4.0	0	2.62	NG	0.000	-	-	-	-	-
			100	2.62	NG	0.000	-	-	-	-	-
			200	2.62	NG	0.000	-	-	-	-	-
			1000	2.62	NG	0.000	-	-	-	-	-

NG=no growth.
The values given in the table are means of three independent determinations.

while all levels of nitrite tested inhibited growth in media containing 2.5 or 4.0% NaCl. As was observed for cultures at 37°C, addition of increasing concentrations of nitrite resulted in increased generation and lag times and decreased exponential growth rates. The inhibitory effect was more pronounced as the pH decreased and the salt level increased.

19°C. Previously (*15*) we have found that *S. flexneri* failed to grow at 19°C in media of pH 5.5. Therefore, experiments with added nitrite were not conducted at pH 5.5. The bacteria grew in media of pH 7.5 at all nitrite levels when NaCl concentrations were 0.5 or 2.5%, but not in the presence of 4.0% NaCl. Bacterial growth was inhibited by 1000 ppm $NaN0_2$ in media of pH 6.5 containing 0.5% NaCl. Growth occurred at pH 6.5 and salt levels of 2.5 and 4.0% only when nitrite was absent.

Even at pH 7.5, lag and generation times were higher and exponential growth rates lower for *S. flexneri* grown at 19°C than for cells grown at 37 or 28°C. Increasing the salt and nitrite levels potentiated the inhibitory effects. Similar results were found for growth at pH 6.5 (Table 2).

A visual summary of the effect of interactions of the variables ($NaNO_2$ and NaCl concentrations, pH, and temperature) on exponential growth rates of *S. flexneri* is shown in Figure 1. Our results indicate that the inhibitory effect of $NaNO_2$ against *S. flexneri* progressively increased as the temperature or pH decreased or the salt concentration was increased. Combinations of these parameters increased the toxicity of nitrite. However, when growth did occur, the maximum population was always high (approximately log 9.0).

TABLE 2. *Gompertz equation parameters and calculated growth curve values for* Shigella flexneri *cultured in BHI medium under various combinations of temperature, pH, sodium chloride, and sodium nitrite concentrations.*

Temp. (° C)	pH	NaCl (%)	$NaNO_2$ (ppm)	A	C	B	M	Growth rate (log_{10} CFU/ml)/h	Generation time h	Lag time h	Max. pop. density log_{10} CFU/ml
37	5.5	0.5	0	2.96	6.39	0.308	4.74	0.72	0.4	1.5	9.4
			100	2.96	6.75	0.156	9.05	0.39	0.8	2.3	9.7
			200	2.96	6.95	0.105	36.81	0.27	1.1	27.3	9.9
			1000	2.96	NG	0.000	-	-	-	-	-
		2.5	0	2.88	6.04	0.213	7.12	0.47	0.6	2.4	8.9
			100	2.90	5.63	0.244	22.82	0.51	0.6	18.7	8.5
			200	2.88	NG	0.000	-	-	-	-	-
			1000	2.88	NG	0.000	-	-	-	-	-
		4.0	0	2.70	5.24	0.169	10.86	0.33	0.9	5.0	7.9
			100	2.70	NG	0.000	-	-	-	-	-
			200	2.70	NG	0.000	-	-	-	-	-
			1000	2.70	NG	0.000	-	-	-	-	-
37	6.5	0.5	0	2.96	6.61	0.475	4.74	1.16	0.3	2.6	9.6
			100	2.96	6.67	0.352	5.64	0.86	0.4	2.8	9.6
			200	2.96	6.45	0.313	6.02	0.74	0.4	2.8	9.4
			1000	2.72	5.94	0.209	30.64	0.46	0.7	25.9	8.7
		2.5	0	2.54	5.75	0.205	5.71	0.44	0.7	0.8	8.3
			100	2.91	5.98	0.211	5.89	0.46	0.6	1.2	8.9
			200	2.91	5.67	0.203	5.65	0.42	0.7	0.7	8.6
			1000	3.00	6.11	0.194	51.61	0.44	0.7	46.4	9.1
		4.0	0	2.95	6.38	0.191	9.57	0.45	0.7	4.3	9.3
			100	2.95	6.25	0.125	13.01	0.29	1.0	5.0	9.2
			200	2.95	5.92	0.122	13.07	0.27	1.1	4.9	8.9
			1000	2.95	NG	0.000	-	-	-	-	-
37	7.5	4.0	0	4.66	4.36	0.308	8.79	0.49	0.6	5.5	9.0
			100	2.78	6.60	0.185	11.05	0.45	0.7	5.6	9.4
			200	2.77	6.61	0.186	11.24	0.45	0.7	5.9	9.4
			1000	2.68	6.70	0.128	12.49	0.32	1.0	4.7	9.4
28	5.5	0.5	0	2.60	7.58	0.138	10.75	0.39	0.8	3.5	10.2
			100	2.85	6.72	0.095	61.76	0.24	1.3	51.3	9.6
			200	2.60	NG	0.000	-	-	-	-	-
			1000	2.60	NG	0.000	-	-	-	-	-
		2.5	0	2.51	6.94	0.108	24.45	0.28	1.1	15.2	9.5
			100	2.91	NG	0.000	-	-	-	-	-
			200	2.89	NG	0.000	-	-	-	-	-
			1000	2.60	NG	0.000	-	-	-	-	-
		4.0	0	2.83	6.67	0.045	38.56	0.11	2.8	16.1	9.5
			100	2.83	NG	0.000	-	-	-	-	-
			200	2.83	NG	0.000	-	-	-	-	-
			1000	2.83	NG	0.000	-	-	-	-	-
28	6.5	0.5	0	2.61	7.29	0.173	8.44	0.46	0.7	2.7	9.9
			100	2.61	7.29	0.155	9.41	0.42	0.7	3.0	9.9
			200	2.61	7.23	0.130	9.89	0.35	0.9	2.2	9.8
			1000	2.87	6.71	0.084	28.28	0.21	1.4	16.4	9.6
		2.5	0	2.60	6.87	0.146	11.12	0.37	0.8	4.3	9.5
			100	2.60	7.21	0.093	15.46	0.25	1.2	4.7	9.8
			200	2.60	7.24	0.088	16.59	0.24	1.3	5.2	9.8
			1000	2.76	6.61	0.081	57.78	0.20	1.5	45.4	9.4
		4.0	0	2.80	6.50	0.097	20.88	0.23	1.3	10.5	9.3
			100	2.80	6.70	0.090	22.79	0.22	1.4	11.7	9.5
			200	2.80	6.70	0.092	23.90	0.23	1.3	13.1	9.5
			1000	2.96	NG	0.000	-	-	-	-	-

DISCUSSION

Results of this study indicate that sodium nitrite can, under certain conditions, inhibit the growth of *S. flexneri*. However, the magnitude of inhibition depends not only on the concentration of sodium nitrite used, but also on other conditions to which the microorganism is subjected, such as temperature, pH, and salt content. The interaction of nitrite with these factors has been reported for other microorganisms (*2,9*).

To our knowledge, the only previously reported study dealing with the effect of nitrite on *Shigella* was that of Fehlhaber (*4*) who studied 21 strains each of *S. flexneri* and *S. sonnei*. He found that for *S. flexneri* the minimum inhibitory concentration of sodium nitrite increased with increasing pH of the medium - from 90 ppm at pH 5.0 to more than 1100 ppm at pH 7.0.

It has been recognized by Tarr (*13*) and later confirmed by others that the antimicrobial activity of nitrite is pH dependent and increases markedly at levels below pH 6. Huntington and Rahn (*7*) showed that the bacteriostatic effect of weak acids, including nitrous acid, is due to the undissociated form of the acid and that the inhibiting concentrations of an acid at all pH values contain the same amount of undissociated acid. Castellani and Niven (*3*) demonstrated this effect using *Staphylococcus aureus*. They showed that the concentration of nitrous acid ($PK_a = 3.4$) for bacteriostasis remains relatively constant (1.7 ppm) throughout the pH range tested (pH 6.90 - 5.05). Thus, as the pH of the medium was lowered one unit, the bacteriostatic effect of added nitrite increased approximately 10-fold.

Growth of *S. flexneri* was progressively inhibited by decreasing temperatures. While Fehlhaber reported (*4*) that all 21 strains of *S. flexneri* studied grew at 10°C, we did not obtain any growth at 10 or 12°C. As reported previously (*15*), low temperatures and high sodium chloride concentrations interacted to decrease growth rates of *S. flexneri* at pH 7.5 to 5.5. In the present study it is evident that sodium chloride magnifies the activity of sodium nitrite against *S. flexneri*. Baird-Parker and Baillie (*1*) suggested that sodium nitrite and sodium chloride are synergistic in inhibiting the growth of *Clostridium botulinum*. Gram-negative bacteria appear to be relatively resistant to the bacteriostatic effect of nitrite (*3*). Schüppel and Krüger (*10*) found that lowering the pH or increasing sodium chloride concentration was more effective than increasing nitrite concentration up to 200 ppm to inhibit the growth of gram-negative bacteria in liquid medium. This observation is in agreement with the results of the present study with *S. flexneri*.

Analysis of the interactions of the parameters studied provides information on changes in growth kinetics as a result of changes in pH, temperature, sodium chloride, or sodium nitrite concentration and will be useful in determining conditions inhibitory to the growth and/or survival of *S. flexneri*. These data, along with the results from other experiments carried out in our laboratory, are being used currently to develop predictive models of the growth of *Shigella* in foods.

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